Effects of Hypoxia on Oxidative Stress, Autophagy and Apoptosis in Cardiomyocytes

Qing-Min Feng1*, Yang Shao1*, Rong Jiao2*, Hong-Wei Wei1*, Ming-Qiang Dai1, Hui-Xing Xie2, Cai-Xia Xu2, Ji-Ke Li1#

1Cardiovascular Center of Hainan Medical University, The First Affiliated Hospital of Hainan Medical University, Haikou, China
2Hainan Medical University, Haikou, China


Received: February 12, 2019 Accepted: April 21, 2019 Published: April 24, 2019

Abstract
Coronary heart disease (CHD) is a hypoxia related disease. However, the relationship of the hypoxia-induced oxidative stress, autophagy and apoptosis in cardiomyocyte remains unclear. In this study, we used CoCl2 to mimic hypoxic conditions in H9c2 cardiomyocytes and study the effects of CoCl2-induced hypoxia on oxidative stress, apoptosis and autophagy, as well as the relationships among these processes. Cell viability and levels of ROS, LC3-II, p62, caspase-3 and PARP were assessed. The viability and morphology of cardiomyocytes were affected by hypoxia, and hypoxia enhanced levels of ROS and the levels of the LC3-II, p62, caspase-3 and PARP proteins in H9c2 cells in a dose-dependent manner. ROS levels rise gradually in the presence of hypoxia; however, it shrinks when hypoxia reaches a certain level. Caspase-3 and PARP levels were raised with the increasing of hypoxia level. Enhanced level of LC3 and decreased levels of p62 in hypoxic cells indicate that autophagy levels are in accord with hypoxia. Based on these results, hypoxia induces oxidative stress, apoptosis and autophagy in cardiomyocytes. Autophagy is a double-edged sword. At a low level, autophagy can resist oxidative stress and protect cardiomyocytes from oxidative stress, while high level autophagy can promote apoptosis of cardiomyocytes.

Keywords
Hypoxia, Oxidative Stress, Autophagy, Apoptosis, Cardiomyocyte

1. Introduction
Coronary heart disease (CHD) is the leading cause of death in older men and women in the western world, and reduced oxygen availability may cause or ex-
acerbate symptoms of CHD [1]. A constant oxygen supply is essential for cardiac viability and function. Hypoxia induces some pathological process, such as oxidative stress, autophagy and apoptosis [2] [3] [4]. To date, researchers have obtained a good understanding of individual pathological processes induced by hypoxia. However, little information is known about the relationships among these processes in cardiomyocytes which limited our understanding of the roles of oxidative stress, autophagy and apoptosis in the progression of human diseases [5].

Oxidative stress is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism [6]. ROS are implicated in irreversible damage to the cell membrane, DNA, and other cellular structures by oxidizing lipids, proteins, and nucleic acids [7]. However, ROS are markers of oxidative stress that are increased under pathological condition [8]. ROS levels are assessed using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Hypoxia inducible factor (HIF) is activated by hypoxia and is a master regulator of oxygen homeostasis, as it regulates the expression of many genes [9]. The expression and activity of the HIF-1α subunit are regulated by cellular oxygen levels, and hypoxia increases HIF-1α levels by inhibiting proline hydroxylation and the subsequent degradation of HIF-1α [9].

Autophagy, an evolutionally conserved process of controlled cellular cannibalization, plays a vital role in cardiac physiology. Perturbations in cardiomyocyte autophagy contribute to the pathogenesis of a wide range of cardiac diseases, many of which culminate in heart failure [10]. LC3 is the only known protein that is specifically associated with all types of autophagic membranes, including phagophores, autophagosomes and autolysosomes [10]. Therefore, the amount of LC3-II correlates well with the number of autophagosomes, which provides a good index of autophagy induction [9]. Levels of p62 have also been used to monitor autophagic flux, and p62 serves as an adaptor protein that links ubiquitinated proteins to the autophagy machinery and facilitates their clearance in the lysosome [11]. Notably, p62 and p62-bound ubiquitinated proteins become incorporated into the completed autophagosomes and are degraded in autolysosomes [12]. Since p62 itself is mainly removed by autophagy, its expression is generally considered to inversely correlate with autophagy activity [12]. The accumulation of p62 has been used as a marker of suppressed autophagy, and similarly, a decrease in the p62 level indicates autophagy activation [11].

Apoptosis is the process of programmed cell death that occurs during heart development [13]. Caspase-3 is a common effector of most apoptotic pathways and is able to cleave several target proteins whose degradation will contribute to the execution phase of the cell death program [14]. Poly ADP-ribose polymerase (PARP) is a conserved nuclear enzyme that is present throughout the phylogenetic spectrum. The precise physiologic role of PARP is to induce DNA repair and maintain genomic integrity [15]. PARP has a complex role in cells induced by DNA damage, and PARP cleavage by caspases is a marker of apoptosis [15].

In patients with CHD, the most effective therapy to reduce ischemic myocardial...
dial injury and infarct size is efficient myocardial reperfusion and early sustained restoration of blood flow (reperfusion) through the occluded coronary artery [16]. However, the myocardial reperfusion process also induces further myocardial cell death, a phenomenon known as myocardial ischemia/reperfusion (I/R) injury [17]. Diverse pathological process, such as oxidative stress, autophagy and apoptosis, contribute to the pathological mechanism underlying CHD [16], So far, researches mainly focus on the single pathological process caused by hypoxia, and there are few studies on the relationship among the three pathological processes [18].

In our study, we used CoCl2 to mimic hypoxic conditions in cardiac H9C2 cells [19] and observed hypoxia-induced oxidative stress, autophagy and apoptosis. We examined the relationships among oxidative stress, apoptosis and autophagy. We believe that an understanding of oxidative stress, autophagy and apoptosis in hypoxic cardiomyocytes and exploring the relationship among the three are important for better clarifying the mechanism of CHD and should have important clinical significance in the prevention and treatment of this disease.

2. Materials and Methods

2.1. Cell Culture

The cardiomyocyte cell line H9C2 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and a 1% antibiotic-antimycotic solution (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO2 [20].

2.2. Hypoxia Induction

The hypoxia model was established using the hypoxia-inducing agent CoCl2, as described in a previous study [20]. Cells were plated in 96-well microtiter plates at a density of 1 × 10^5 cells/ml and cultured with DMEM supplemented with 10% FBS for 6 h at 37˚C. Cells were subsequently incubated in serum-free CO2-independent DMEM supplemented with various concentrations of CoCl2 (300, 600, 900 or 1200 µM) for 16 h. Normoxic control cells were incubated under the same conditions in a normal atmosphere. Normoxic cells received normal serum and no CoCl2 treatment, and the hypoxic cells were then incubated in a hypoxic chamber.

2.3. Cell Viability Assessment under Various Hypoxic Conditions

MTT was added at a final concentration of 25 mg/ml. After a 4-h incubation at 37˚C, the reaction was halted by adding 150 µl of dimethyl sulfoxide (DMSO), and the relative absorbance value (AV) was measured at 595 nm using a microplate reader [21]. Cell viability was calculated according to the AV and density of cells.

2.4. Western Blot Analysis

Cells were lysed with radioimmunoprecipitation assay buffer [50 mM Tris-HCl...
(pH 7.4), 150 mM NaCl, 0.1% SDS, 1% NP-40, and protease inhibitor cocktail] for 30 min and centrifuged at 12,000 × g for 10 min prior to supernatant collection. The protein concentration was quantified using the bicinchoninic acid assay. Equal amounts (60 µg) of protein were loaded into each lane of 10% SDS-PAGE gels, separated, and then transferred to polyvinylidene fluoride (PVDF) membranes (Roche Diagnostics GmbH, Mannheim, Germany). After blocking with 5% fat-free milk in Tris-buffered saline-Tween-20 (TBST) for 1 h at room temperature, PVDF membranes were incubated with primary antibodies (1:2000) overnight at 4˚C. Membranes were washed with TBST, incubated with horseradish peroxidase-conjugated anti-rabbit (L3012, Signal way Antibody, Nanjing, China) or anti-mouse (L3032, Signal way Antibody) secondary antibodies in TBST (1:5000) for 1 h at room temperature, and then visualized using a super enhanced chemiluminescence detection reagent (Beyotime Institute of Biotechnology, Haimen, China). Signals were detected using an Image Station 4000 R (Kodak, Rochester, NY, USA). Results were quantified using ImageJ software version 1.44 (National Institutes of Health, Bethesda, MD, USA). Each experiment was repeated separately at least three times.

2.5. Fluorescence Assay of Intracellular ROS Levels

Intracellular ROS generation was determined using the ROS-dependent oxidative conversion of cell-permeable DCFH-DA dye to the fluorescent dye dichlorofluorescein. H9C2 cells were treated as described above, washed 3 times with PBS, and incubated with a 10 µM DCFH-DA solution in serum-free medium at 37˚C for 30 min in the dark. Cells were washed 3 times with PBS, and ROS concentrations were quantified using a ZEISS LSM 510 confocal microscope at a wavelength of 488 nm.

2.6. Statistical Analysis

All data are presented as means ± SD. Data were analyzed using SPSS software version 19.0. Statistically significant differences were determined using one-way analysis of variance (ANOVA) with the LSD post hoc test was performed. Notably, P < 0.05 was considered to indicate a statistically significant difference [21][22].

3. Results

3.1. Effects of Hypoxia on the Viability of H9C2 Cells

Based on the results from the MTT assay, the CoCl2 treatment decreased cell viability in a concentration-dependent manner (Figure 1). Cell viability decreased after a 16 h incubation with increasing concentrations of CoCl2 (300, 600, 900, or 1200 µM) compared with the control group (P < 0.05), and 600 µM was sufficient to decrease viability by ~50%. At a concentration of 300 µM, CoCl2 began to exert cytotoxic effects, and cell viability gradually decreased as the CoCl2 concentration increased, reaching a minimum at 1200 µM (P < 0.05).
3.2. Effects of Hypoxia on the Morphology of H9C2 Cells

Cells in the control group were spindle-shaped, the cell membrane was smooth, grew well, displayed homogeneous adhesion, and no floating and vacuolated cells were observed (Figure 2). In the treatment group, fewer cells survived, and apoptotic cells with irregular cell membranes were observed. The number of cells displaying this phenotype increased as the CoCl$_2$ concentration increased. After treatment with 300 μM CoCl$_2$, the cell morphology changed, but the cell membrane was still smooth, and only a small number of cells failed to adhere and were floating in the culture bottle. At a concentration of 600 μM CoCl$_2$, significantly fewer adherent cells were observed compared to cells treated with lower concentrations of CoCl$_2$, and the number of floating cells increased. When the CoCl$_2$ concentration reached 900 - 1200 μM, the number of adherent cells was further reduced, and the morphological integrity of the membrane of adherent cells was further reduced.

3.3. Effects of Hypoxia on Oxidative Stress in H9C2 Cells

As shown in Figure 3, after a CoCl$_2$ treatment for 16 h, the cells were stained with the fluorescent dye. The treatment group exhibited a significant increase in fluorescence compared with the control group. In cells treated with 0 - 600 μM CoCl$_2$, the fluorescence intensity gradually increased, suggesting that the level of oxidative stress increased. The highest fluorescence intensity was observed in cells treated with 600 μM CoCl$_2$, and the value continued to increase as the concentration of the treatment increased; however, the intracellular ROS levels decreased ($P < 0.05$), suggesting that intracellular oxidative stress decreased.
Figure 2. Cells were cultured with CoCl$_2$ for 16 h. A-E Cells were incubated with CoCl$_2$ concentrations of 0, 300, 600, 900 or 1200 μM. Images of cell morphology are shown. A significant difference was not observed in cells treated with 300 μM CoCl$_2$. As the concentration increased, the number of adherent cells decreased, floating cells and cell vacuolization were observed, and the cell morphology was significantly altered. Scale bar = 100 μm.

3.4. Effects of CoCl$_2$ on H9C2 Cells

The expression of HIF-1α in H9C2 cells was detected by western blotting. As shown in Figure 4, after a 16 h treatment, a significant difference was not observed between the 300 μM and control group. When the concentration of CoCl$_2$ increased to 600, 900 and 1200 μM, HIF-1α levels were significantly increased in a concentration-dependent manner ($P < 0.05$).

3.5. Effects of Hypoxia on the Apoptosis of H9C2 Cells

The expression of caspase-3 and PARP in H9C2 cells were detected by western blotting. As shown in Figure 5, significant differences in the levels of these proteins were not observed between cells treated with 300 μM CoCl$_2$ and the control group. When the concentrations of CoCl$_2$ in the treatment groups were 600, 900 and 1200 μM, caspase-3 levels were significantly increased in treated cells in a concentration-dependent manner compared with the control group ($P < 0.05$). Significant differences in PARP levels were observed in cells treated with 900 and 1200 μM CoCl$_2$ ($P < 0.05$).
Figure 3. The intracellular ROS content was determined in cells. A-E Cells were incubated with CoCl₂ concentrations of 0, 300, 600, 900 or 1200 μM. Extremely low and almost unpredictable ROS levels were detected in the control group. As the CoCl₂ concentration increased, the ROS content increased gradually, and the highest levels were observed in cells treated with 600 μM CoCl₂. When the CoCl₂ concentration continued to increase, the ROS content gradually decreased. *A statistically significant difference compared with the control group (P < 0.05). Scale bar = 100 μm.

Figure 4. The degree of hypoxia was observed on H9C2 cells. As the CoCl₂ concentration gradually increased, the HIF-1α level was not statistically significantly different between cells treated with 300 μM CoCl₂ and control cells, whereas the HIF-1α level was significantly increased in cells treated with ≥600 μM CoCl₂. *A statistically significant difference compared with the control group (P < 0.05).
Figure 5. The levels of caspase-3 and PARP in cells were tested. Caspase-3 levels in cells treated with 300 μM CoCl₂ were not significantly different from control cells, but the caspase-3 content increased in cells treated with ≥600 μM CoCl₂. PARP levels were not significantly different between cells treated with 300 μM and 600 μM CoCl₂, but PARP levels increased in cells treated with 900 μM CoCl₂ in a concentration-dependent manner.

* A statistically significant difference compared with the control group (P < 0.05).

### 3.6. Effects of Hypoxia on Autophagy in H9C2 Cells

The levels of LC3-II and p62 in H9C2 cells were detected by western blotting. As shown in Figure 6, after a 16 h treatment, the levels of these proteins were not significantly different between cells treated with 300 μM CoCl₂ and the control group. In cells treated with 600, 900 and 1200 μM CoCl₂, the LC3-II levels were significantly increased (P < 0.05), while the level of p62 decreased as the concentration increased, suggesting that the autophagy was induced to a statistically significant level (P < 0.05).

### 4. Discussion

CHD is the leading cause of cardiovascular mortality worldwide, with over 4.5 million deaths occurring in the developing world [23]. CHD is a multifactorial heritable disease caused by a variety of risk factors related to the oxygen and energy needs of the myocardial tissue [24]. However, in patients with CHD, the exact relationships among oxidative stress, autophagy and apoptosis have not been confirmed. Moreover, researchers have not yet determined whether other pathological processes occur in response to hypoxia. To date, few reports have examined the relationships among these three processes. We believe that a better understanding of these relationships will facilitate the prevention and treatment of CHD. In our study, we treated H9C2 cells with CoCl₂ to mimic cardiomyocyte hypoxia. Hypoxia affected cell viability and growth, based on the results from the MTT assay and microscopic observations. Hypoxic cells also exhibited increased HIF-1α and ROS levels, confirming that hypoxia induced oxidative stress. Additionally, caspase-3 and PARP levels were increased, indicating that hypoxia induced apoptosis. Simultaneously, intracellular LC3-II levels increased and p62 levels decreased, confirming that hypoxia induced autophagy in hypoxic cells. Based on the results of these experiments, hypoxia induced oxidative stress,
apoptosis and autophagy in H9C2 cells. Based on these results, hypoxia induces oxidative stress, apoptosis and autophagy in cardiomyocytes. Autophagy is a double-edged sword. At a low level, autophagy can resist oxidative stress and protect cardiomyocytes from oxidative stress, while high level autophagy can promote apoptosis of cardiomyocytes.

Hypoxia inhibits HIF-1α hydroxylation, thereby promoting its accumulation; hypoxia also allows the mitochondria to release more ROS into the cytosol [25]. In our study, both ROS and HIF-1α levels were increased in the treatment group, indicating that we successfully induced oxidative stress in hypoxic cells using CoCl₂. Increased ROS production leads to DNA, protein and lipid modifications, and activates stress-signaling pathways leading to heart failure in subjects with I/R injury [26]. Several studies have reported the occurrence of both autophagy and oxidative stress in response to cell death stimuli. However, very little experimental evidence for a direct role for autophagic cell death in ROS/RNS- or oxidative stress-mediated toxicity has been reported [5]. For example, oxidative stress was reported to induce cell death via both autophagy and necrosis, and knockdown of Atg7 (Atg7 is a core autophagy protein [27]) only delayed early cell death progression in primary murine cortical neurons [28]. In contrast, a number of recent studies have reported a protective effect of autophagy on oxidative stress-induced cell death [5]. For example, lipocalin-2 (NGAL) attenuates autophagy to exacerbate cardiac apoptosis induced by myocardial ischemia [29]. Oxidative stress is associated with increased formation of ROS that contribute to the pathophysiology of I/R injury. Myocardial I/R injury must be prevented to achieve a successful CHD surgery and recovery. Novel treatments that inhibit or ameliorate the disease process must be developed to allow sufficient time for subsequent treatment.
Autophagy acts as a survival mechanism under stress conditions, maintaining the cellular integrity by regenerating metabolic precursors and clearing subcellular debris [27]. This process contributes to basal cellular and tissue homeostasis, regulates development in higher organisms, and can affect pathogenesis [27]. During autophagy, autophagosomes fuse with lysosomes to form autolysosomes, and the autophagosome content is degraded by lysosomal hydrolases. LC3 in the autolysosome lumen is simultaneously degraded [30]. Therefore, an increase in LC3 levels may result from a reduced turnover of autophagosomes rather than from increased autophagy activity, and p62 is known to be degraded together with the autophagosome content [30]. In our study, LC3-II levels were increased concomitantly with decreased p62 levels, indicating that autophagy was induced by the CoCl$_2$ treatment in a concentration-dependent manner, consistent with other studies [31]. Autophagy may exhibit dual functions in hypoxia-induced cell damage. In most cases, the activation of autophagy protects the cell; however, excessive activation of autophagy may also mediate cell death through a process known as autophagic cell death (ACD) [32]. Nevertheless, the activation of autophagy has also been reported to induce apoptosis, and some proteins that play important roles in autophagy may also induce the apoptosis signal transduction pathway [32].

Apoptosis, a form of programmed cell death, occurs in a wide range of physiological and pathological situations. It is characterized by cell shrinkage, programmed DNA degradation, and increased expression of caspase-3 [33]. In our study, the expression of caspase-3 increased as the CoCl$_2$ concentration increased, suggesting that the degree of hypoxia was positively correlated with the level of apoptosis. However, a limitation of cleaved caspase-3 was not detected to confirm that hypoxia induced the activation of caspase-3. PARP is a critical enzyme involved in the repair of DNA strand breaks [34]. In the present study, PARP levels increased in the presence of higher concentrations of CoCl$_2$. Oxidative stress-induced production of ROS likely damages the DNA and increases intracellular PARP expression. Excess ROS also induce the oxidative modification of cellular macromolecules, inhibit protein function, and induce cell death [35]. In our experiment, the highest ROS levels were observed in cells treated with the IC50 concentration of CoCl$_2$, and the ROS level decreased at higher concentrations, which may be related to the increased levels of apoptosis and autophagy, which reduced ROS production. The relationship between autophagy and apoptosis is complex, as they share the same set of cellular regulatory proteins and are closely linked. Autophagy has been reported to inhibit or delay the occurrence of apoptosis, as well as to promote apoptosis [19]. In fact, the role of autophagy itself is two-way, that is, to protect or promote the damage depending on experiments, as shown in the present study, excess autophagy likely promoted the apoptosis of cardiomyocytes and this is only based on results for marker proteins of autophagy and apoptosis, while the actual occurrence of autophagy and apoptosis requires confirmation using additional experiments.
In our study, cell hypoxia was simulated by the CoCl₂ treatment, and cells cultured under hypoxic conditions exhibited oxidative stress, autophagy and apoptosis. As the CoCl₂ concentration increased, the levels of autophagy and apoptosis gradually increased, suggesting that both processes may employ a common molecular mechanism or signaling pathway. Moreover, when the concentration of CoCl₂ was less than 600 μM, the ROS level increased as the CoCl₂ concentration increased, but a significant difference in the increase in caspase-3 levels was not observed. Thus, we speculated that intracellular antioxidants such as glutathione might block ROS-induced apoptosis, and the cells are still in the negative feedback regulation stage. However, when the concentration of CoCl₂ exceeded 600 μM, the level of apoptosis is increased, but the ROS content was not increased. Therefore, treatment with high doses CoCl₂ likely influenced the intracellular sources from which ROS were produced through effects on the mitochondrial integrity and electron transfer chain, and may have even directly caused cell death to decrease the ROS content. Based on these findings, oxidative stress may not be the most important factor contributing to apoptosis. We also noticed that at CoCl₂ concentrations less than 600 μM, oxidative stress occurred, but the LC3-II level did not increase, potentially due to a cellular reduction mechanism that blocks the transduction of ROS signals. When the concentration exceeded 600 μM, the ROS content decrease and the level of autophagy increased. We speculated that autophagy may function as an antioxidant and increase the phagocytosis of oxidative stress-related oxidases and organelles, such as NADPH oxidase, mitochondria and other structures, in vivo to decrease the ROS content decreased. In this experiment, when the concentration of CoCl₂ was less than 600 μM, the levels of autophagy- and apoptosis-related proteins were not increased, and when the concentration of CoCl₂ exceeded 600 μM, the levels of these proteins increased. We also confirmed that CoCl₂ mimicked cardiomyocyte hypoxia by inhibiting cell growth, and inducing oxidative stress, apoptosis and autophagy. Moreover, when the concentration of CoCl₂ was 600 μM, the maximum level of intracellular oxidative stress was observed. As the level of hypoxia increased, cells may have alleviated intracellular oxidative stress by increasing autophagy; however, when the concentration of CoCl₂ continued to increase, cells were more likely to undergo apoptosis following autophagy than after oxidative stress in our opinion. Hypoxia-induced oxidative stress leads to irreversible damage in many diseases characterized by ischemia. Treatments that decrease ROS production or increase the ROS scavenging ability of the system may exert beneficial effects on the disease. Endogenous mechanism or exogenous intervention can coordinate the function of autophagy and inhibit the damage of oxidative stress and apoptosis to myocardium.

The relationship among oxidative stress, autophagy and apoptosis is complex, and mutual influence is achieved through multiple signaling systems. Further researches are needed to verify the changes in cellular and molecular signals of oxidative stress, autophagy and apoptosis in the heart.
Acknowledgements

This work was supported by grants from the National Natural Sciences Foundation of China (81200150 and 81660064), Hainan Province Natural Science Foundation (20158338), Hainan Provincial Foundation for Health Department (14A210227), Cultivating Fund of Hainan Medical University (HY2014-007), Student’s Innovative Project of Hainan Medical College (HYCX2015003), and National Students’ Innovation and Entrepreneurship Training Program (201611810052).

Conflicts of Interest

The authors declare no conflict of interest.

References


the Medical Faculty of Palacký University, Olomouc, Czech Republic, 157, 340-345. https://doi.org/10.5507/bp.2013.061


